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**REMARKS**

In accordance with the present invention, there are provided methods for the identification of active proteins in a complex protein mixture (*e.g.*, a proteomic mixture). Following reaction of a complex protein mixture with one or more activity based probes (ABPs), the resulting protein conjugates are proteolytically digested to provide probe-labeled peptides. While the skilled artisan would have expected, prior to the present invention, that proteolytic digestion would lead to a more complex protein mixture, in accordance with the present invention, it has been demonstrated that such proteolysis can actually provide an advantageous simplification of the complex protein mixture during subsequent analysis.

In preferred embodiments, ABPs are selected such that each active target protein forms a conjugate with a single ABP, most preferably at a single discrete location in the target protein; thus, each conjugate gives rise to a single ABP-labeled peptide. Enrichment separation, or identification of one or more ABP-labeled peptides may be achieved using liquid chromatography and/or electrophoresis. Additionally, mass spectrometry may be employed to identify one or more ABP-labeled peptides by molecular weight and/or amino acid sequence. In particularly preferred embodiments, the sequence information derived from one or more of the ABP-labeled peptide(s) is used to identify the protein from which the peptide originally derived. Variations of these aspects can involve the comparison of two or more proteomes, *e.g.*, with ABPs having different ligands, or, when analysis comprises mass spectrometry, having different isotopic compositions.

Invention methods provide enhanced simplicity and accuracy in identifying changes in active protein composition of a complex protein mixture. Using ABPs that bind to active target proteins, the analysis of complex protein mixtures may be greatly simplified, particularly by providing ABPs that bind to active target proteins at a single site. The proteins are then proteolytically digested, resulting in a single representative ABP-labeled peptide fragment from each of the conjugates. Using various approaches to identification of the ABP-labeled peptide(s), the protein(s) from which each ABP-labeled peptide originally derived can be identified.

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By the present communication, claims 21 and 48 have been amended to define Applicant's invention with greater particularity. No new matter is introduced by the subject amendments as the amended claim language is fully supported by the specification and original claims.

Upon entry of the amendments submitted herewith, claims 1-48 will remain pending, with claims 21-32 and 48 under active prosecution. The present status of all claims in the application, and current amendments thereto, are provided in the Listing of Claims presented herein beginning on page 2.

The rejection of claims 21, 22, 24, 25, 27, 28 and 48 under 35 U.S.C. § 102(e) as allegedly being anticipated by Cravatt et al., US 2002/0045194, is respectfully traversed. Applicant's invention, as defined, for example, by claim 21, distinguishes over Cravatt by requiring a method for determining the presence, amount, or activity of one or more active target proteins in a complex protein mixture, the method comprising:

- (a) contacting the complex protein mixture with a single activity based probe that specifically binds predominantly to a single target site on one or more active target proteins;
- (b) proteolyzing the active target protein(s) to produce a product mixture;
- (c) separating the product mixture into one or more components, one or more of which comprise peptides bound to the probe; and
- (d) generating a signal from the peptides bound to the probe, wherein the signal is correlated to the presence, amount, or activity of the one or more active target proteins in the complex protein mixture.

Cravatt does not disclose such a method. Instead, Cravatt requires the use of sets of probes (e.g., a light probe and a heavy probe; see paragraph [0128] at page 14 of Cravatt) for the analysis contemplated therein. The ultimate analysis thereof according to Cravatt also requires simultaneous analysis of samples treated with the sets of probes by mass spectrometry.

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The rejection of claims 21-28, 30-32 and 48 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Aebersold et al., US 2002/0076739, in view of Cravatt, is respectfully traversed. Applicant's invention, as defined, for example, by claim 21, distinguishes over the combination of Aebersold in view of Cravatt by requiring a method for determining the presence, amount, or activity of one or more active target proteins in a complex protein mixture, the method comprising:

- (a) contacting the complex protein mixture with a single activity based probe that specifically binds predominantly to a single target site on one or more active target proteins;
- (b) proteolyzing the active target protein(s) to produce a product mixture;
- (c) separating the product mixture into one or more components, one or more of which comprise peptides bound to the probe; and
- (d) generating a signal from the peptides bound to the probe, wherein the signal is correlated to the presence, amount, or activity of the one or more active target proteins in the complex protein mixture.

Aebersold does not disclose or suggest such a method. Instead, as acknowledged by the Examiner, "Aebersold et al differ from the instant invention in failing to teach the probe is an activity based probe." (See page 4, lines 10-11 of the Office Action). This is a very significant difference for a variety of reasons. For example, activity based probes label a single target site on each protein, thus following a proteolytic digest, only a single labeled peptide will be present from each protein. Prior to the present invention, a standard belief in the mass spectrometry community for identification of proteins was that a single peptide could not give data with high enough confidence to unambiguously identify a protein through automated sequence searching algorithms. When only a small number of peptides were found to match a particular protein (1-3 peptides) researchers manually inspected the MS data and determined whether it was valid or not. See, for example, Florens L et. al., Nature 419:520-526, October 3, 2002 (especially the final paragraph of the methods section, which discusses the analysis of "low coverage loci" by visual inspection), Adkins JN et. al., Molecular and Cellular Proteomics 2002, December, pp.

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947-955 (especially the legend of Table I legend at page 949, which expressly asserts that “When three or fewer peptides for an individual protein passed the criteria shown in Table I, the mass spectra for those peptides were inspected manually.”), and Washburn MP et. al., *Nature Biotechnology*, 2001, vol 19, pp 242-247 (especially the final paragraph of the experimental section, which states that “We manually confirmed each SEQUEST result from every protein identified by four or fewer peptides”).

Since a typical mass spectrometry run generates on the order of 4000 spectra, manual analysis (at a rate of about 15 minutes per spectrum) would not be feasible if all data was expected to fall into the category of single site labeling of each protein.

In addition, since activity based probes label only a single site on each target, a proteolytic digest step employing ABPs does not increase the complexity of the sample as it normally would using probes such as those described in Aebersold. In contrast, the probes described by Aebersold label multiple sites on each target protein (typically 5-30 sites—if, for example, the Aebersold “protein reactive group” is a cysteine, since the average protein in the complete human database has about 360 amino acids and cysteine has a relative abundance of 2.8%, the average number of cysteine residues per protein is 10).

Thus when a labeled sample according to Aebersold is digested, the number of labeled peptide species is substantially increased, typically at least 10X versus the number of labeled proteins. This property of ABPs enables the use of separation methods that are not applicable to the Aebersold methods, e.g., lower resolution, higher throughput separation methods such as CE or LC (instead of LCMS/MS typically used for the Aebersold methods).

Furthermore, activity based probes are typically larger than the probes used by Aebersold et al, and the labeling sites of the probes (particularly for serine hydrolase probes) tend to result in very large peptides (up to 7800 Da). This results in the ability to collect mass spectrometry

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data only on higher charge states (+3, +4, +5) of peptide ions. Prior to the present invention, there was a belief in the field of mass spectrometry-based proteomics that mass spectrometry data with sufficient quality to give a confident peptide sequence could only be obtained from +1, +2, and in some cases +3 ions. Indeed, most laboratories only attempted to identify peptide sequences from +1 and +2 peptide data (see, for example, Aebersold RA and Goodlet DR, Chemical Reviews, 2001, vol. 101, pp 269-295 (the first paragraph on page 278 states that "[M+2H]<sup>2+</sup> ions of peptides will produce tandem mass spectra of higher quality than those from either [M+H]<sup>+</sup> or [M+3H]<sup>3+</sup> peptide ions. The [M+2H]<sup>2+</sup> peptide ions fragmented under low-energy CID produce spectra...that are more readily interpreted than tandem mass spectra of [M+3H]<sup>3+</sup> and higher charge states"); Washburn MP et. al., Nature Biotechnology, 2001, vol 19, pp 242-247 (the final paragraph of the experimental section states "Peptides identified by SEQUEST may have three different charge states (+1, +2, +3), each of which results in a unique spectrum for the same peptide."); Shen Y et. al., Analytical Chemistry, 2004, 76, pp 1134-1144 (see the experimental section which provides the score criteria used for +1, +2, and +3 ions. Charge states higher than +3 were not searched); Florens L et. al., Nature 419, October 3, 2002, pp 520-526 (see the experimental section thereof which provides the score criteria used for +1, +2, and +3 ions. Charge states higher than +3 were not searched); and Adkins JN et. al., Molecular and Cellular Proteomics 2002, December, pp. 947-955 (see the experimental section thereof which provides the score criteria used for +1, +2, and +3 ions. Charge states higher than +3 were not searched).

Moreover, since each protein leads to a single species when using the current method with ABPs, it is not necessary to know the origin of each peptide (i.e., the sequence of peptide determined by mass spectrometry) in order to determine the number of protein species changing between samples. For example if a CE separation were performed on a sample labeled by the probes described in the Aebersold et al method, and 10 peaks were observed to change, one would not know whether 10 proteins had changed levels or if a single protein with 10 different labeling sites had changed. In contrast, in accordance with the invention method employing

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ABPs, the number of peptides is roughly equal to the number of protein labeled in the original, undigested sample.

Still further, the specific labeling sites of activity based probes can be accurately predicted in most cases (particularly for serine hydrolase, cysteine hydrolases, and protein kinases), thus the scope of peptides that *could* be present in a digested, ABP-labeled sample can be analyzed *in silico* (computationally). Indeed, even within families of highly related enzymes (such as the trypsin family of serine proteases, protein kinases, cysteine proteases, and the like), the peptides derived from tryptic digests of ABP labeled proteins have significant differences in their amino acid sequences that enable separation by standard chromatographic methods, and/or their clear identification by mass spectrometry. Generally >95% of such peptides are non-redundant, i.e., the particular sequence is not shared by any other protein. This is neither disclosed or suggested by the cited prior art, and is a key realization for the success or general functionality of the claimed method.

Yet another prejudice in the art which Applicant had to overcome to arrive at the present invention was the fact that, at the time of the present invention, the ability to obtain consistent proteolytic digests in a multitude of proteomic mixtures had not been described or validated in the art. In fact, nearly all published proteolytic digest procedures suggested using an amount of protease (trypsin) equal to a particular fraction of the amount of protein in the sample (e.g., 1 mg trypsin per 20 mg protein). See, for example, Adkins JN et. al., Molecular and Cellular Proteomics 2002, December, pp. 947-955 (especially the experimental section at page 948 which states that samples were "digested with trypsin 1:50 (w/w) ratio for 2 h") and Shen Y et. al., Analytical Chemistry, 2004, 76, pp 1134-1144 (especially the experimental section, at page 1136, which states that the "protein was enzymatically digested using sequencing-grade modified porcine trypsin at a ratio of 1:50 (w/w)").

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Thus researchers in the field at the time of the present invention believed that under such conditions trypsin was exhibiting first order kinetics (i.e., the rate of proteolysis was dependent only on the trypsin concentration, and the trypsin active site was saturated with substrate). If this were true, a precise determination of protein concentration would be required for every sample, hampering the throughput and increasing the sample requirements for such a method. Further the actual protein content (type of proteins present) would impact the overall hydrolysis rate and make it impossible to obtain consistent digests over a broad range of sample types.

Contrary to the pre-conceived notions in the art, in accordance with the present invention, it has been established that trypsin in fact operates under second order kinetics at the typical protein concentrations used in proteomics experiments (0.2-50 mg/mL). Thus when more protein is presented to the enzyme, it increases its turnover rate. Therefore, contrary to the pre-conceived notions in the art, a constant amount of trypsin can be added to any sample, and the time required to reach a particular degree of proteolysis (e.g., 99% cleaved) is constant regardless of the protein concentration of origin of the proteomic sample.

Further reliance on Cravatt is unable to cure the deficiencies of Aebersold. Indeed, it is respectfully submitted that no motivation has been provided to combine the cited references. While Aebersold relates to methods for quantitative analysis, Cravatt relates to proteomic analysis. It is only with improper hindsight analysis, having benefit of Applicant's specification, that the asserted combination of references can be applied to the present claims.

The rejection of claim 29 under 35 U.S.C. § 103(a) as allegedly being unpatentable over Aebersold et al. and Cravatt, and further in view of Little et al., US 2003/0003465, is respectfully traversed. Applicant's invention, as defined, for example, by claim 29, distinguishes over the combination of Aebersold and Cravatt in view of Little, by requiring a method for determining the presence, amount, or activity of one or more active target proteins in a complex protein mixture, the method comprising:

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- (a) contacting the complex protein mixture with a single activity based probe that specifically binds predominantly to a single target site on one or more active target proteins;
- (b) proteolyzing the active target protein(s) to produce a product mixture, wherein prior to proteolyzing, the one or more active target proteins bound to the probe are bound to a solid support;
- (c) separating the product mixture into one or more components, one or more of which comprise peptides bound to the probe; and
- (d) generating a signal from the peptides bound to the probe, wherein the signal is correlated to the presence, amount, or activity of the one or more active target proteins in the complex protein mixture.

As discussed above, neither Aebersold nor Cravatt, taken alone or in combination, are capable of rendering obvious the present invention. Indeed, as acknowledged by the Examiner, "Aebersold et al and Cravatt et al differ from the instant invention in failing to teach prior to the proteolyzing step, the [sp—that] one or more active target protein[(s)] bound to the probe are bound to a solid support." (See page 5, lines 16-18 of the Office Action).

Further reliance on Little is unable to cure the deficiencies of the primary references. Indeed, it is respectfully submitted that the asserted combination of references can only be advanced with improper hindsight analysis, having benefit of Applicant's specification.



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In view of the above amendments and remarks, reconsideration and favorable action on all claims are respectfully requested. In the event any issues remain to be resolved in view of this communication, the Examiner is invited to contact the undersigned by telephone so that a prompt disposition of this application can be achieved.

Respectfully submitted,

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